Population genetics of *Delissea waianaeensis*

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Final Report

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FINAL REPORT

Population genetics of *Delissea waianaeensis* Lammers (Campanulaceae) and parental identification of a potential new founder individual;

and

Identification of invasive plant species on U.S. Army lands, 2008-2009.

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Population genetics of *Delissea waianaeensis* Lammers (Campanulaceae) and parental identification of a potential new founder individual

Introduction

The Hawaiian endemic species *Delissea subcordata* (*oha*) in the Campanulaceae family was federally listed as endangered in 1996 (Russell & Bruegman 1996). Subsequent taxonomic revision of the species (Lammers 2005) has found that the taxon comprises two extant species on O‘ahu, namely the two subspecies of *D. subcordata*, historically found within the Ko‘olau Mountains; and *D. waianaeensis* found along the Wai‘anae Mountain range. The two species differ in foliar and floral characters, most notably anther length. The endangered species listing of *D. subcordata* actually pertains to *D. waianaeensis* (Lammers 2005).

The U. S. Army Natural Resources Staff is responsible for maintaining the stability of *Delissea waianaeensis* on O‘ahu, and is actively reintroducing populations of *D. waianaeensis* in areas that historically contained the species. One reintroduction population in the Kahanahāiki area was started from individuals from the wild Kapuna population located approximately 1800 meters away, using KAP-A and KAP-B stock. A second wild population is located in Pahole about 850 meters away. The reintroduced population was started from founders located in these two gulches, and reintroduced plants have been documented as having flowered and fruited, and seeds have been collected from these individuals. In 2000, a new recruit was found approximately 100 meters from the reintroduced plants. It is unclear whether this individual is an F_1 from a reintroduction parental source or from the seed bank of the historical population. The goal of this project is to determine the potential parental source of this natural recruit and to provide an indication of the within- and between-population genetic variability of the species.

Materials and Methods

Leaf material was collected by Army Natural Resources Staff from 24 shade-house-grown individuals representing the seven distinct wild population units of *D.waianaeensis*: Kapuna, Pahole, ‘Ēkahanui, Mohiākea, Palikea Gulch, Kalua‘ā, Keālia, and Palawai (Table 1, Figures 1 & 2). Each population has just one or a few wild founders remaining. Leaf tissues were refrigerated until DNA extraction could be undertaken then rapidly dehydrated in silica gel and frozen at 80C for future molecular analyses. Tissues and extracted DNA were accessioned into Bishop Museum collections (accession number 2008.174). Genomic DNA was extracted from 6-10 mg dried plant material using a DNeasy Plant Mini Kit (QIAGEN Inc.) following the recommended protocol. Extracted DNA has been stored at 80C.

Table 1: Specimens included in analyses, with source plants ordered from north to south geographic localities in the Waianae Mountains of the wild founder. Sample Name is the US Army PopRefSiteID; PCMB No. is the collection number of the tissues and DNA aliquots stored at the Bishop Museum. Samples marked with * were removed from statistical analyses.

Population	Sample Name	PCMB No.
Keālia	LIA-A2*	4405
Unknown parentage	MMR-C1	4415
Pahole	PAH-A1*	4406
	PAH-A2	4407
	PAH-B2	4408
	PAH-B3	4409
	PAH-E1	4410
Kapuna	KAP-A1	4403
	KAP-A5	4404
	KAP-C2	4402
Palikea	ALI-A2*	4392
	ALI-B3	4395
	ALI-B5	4393
	ALI-B6	4394
South Mohiākea	SBW-A1	4413
	SBW-A3	4411
	SBW-A4	4412
Kalua‘ā	KAL-B1	4401
‘Ēkahanui	EKA-A1	4396
	EKA-A4	4397
	EKA-A7	4398
	EKA-A10*	4399
	EKA-B1	4400
Palawai	PAL-A1	4414

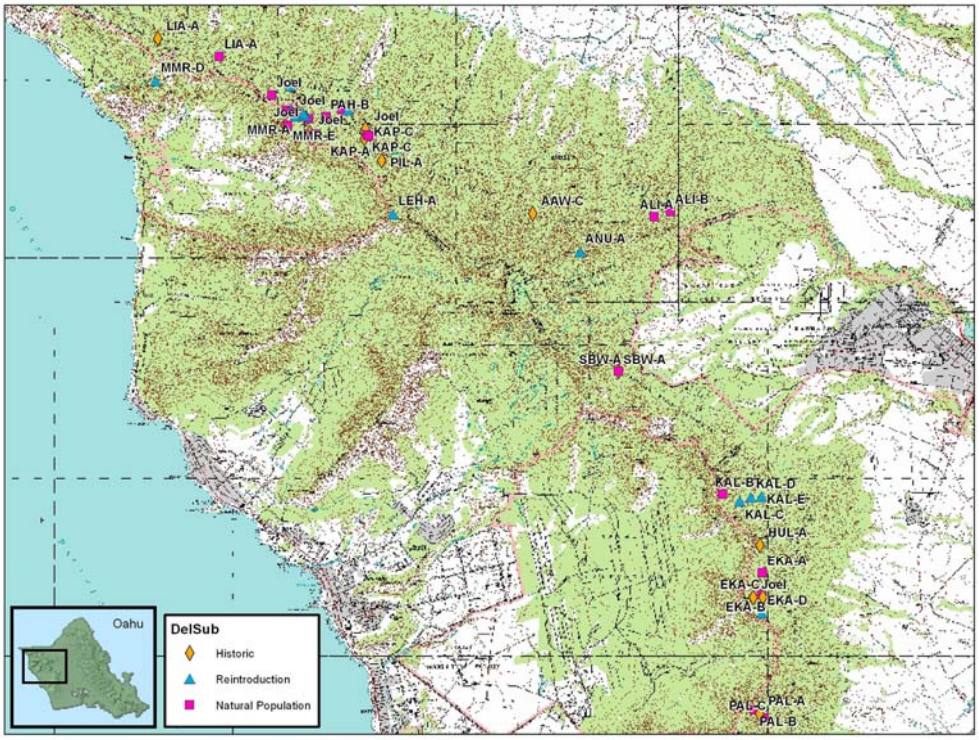

Figure 1: Distribution of population units of *Delissea waianaeensis* on O‘ahu.

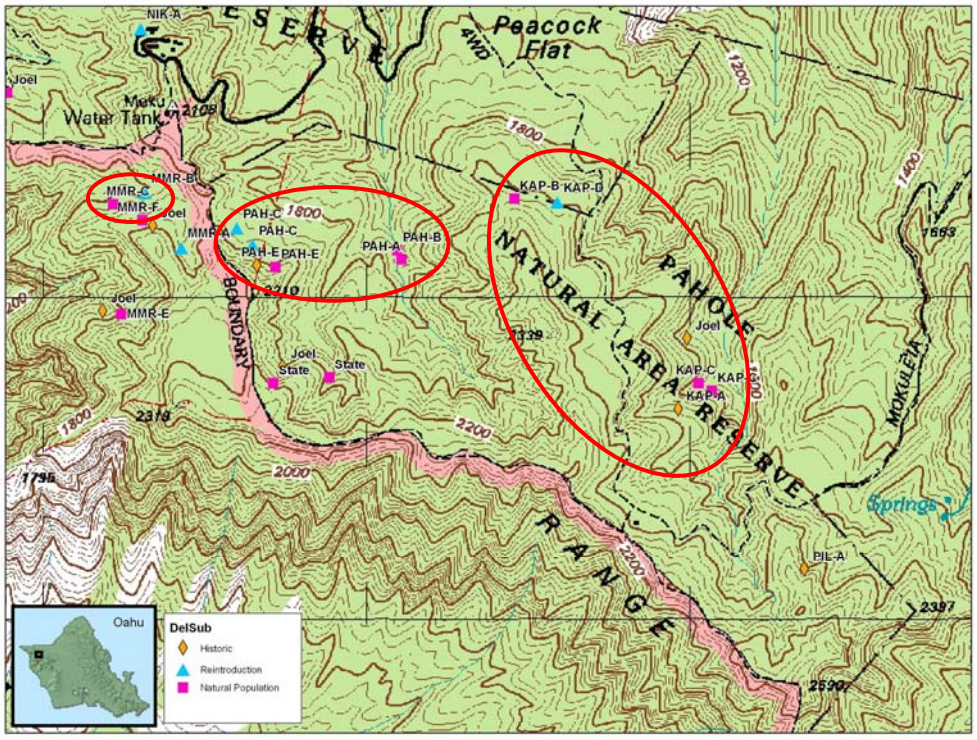

Figure 2: Location of the Kahanahāiki individual (MMR-C1) in relation to wild and introduced populations of *Delissea waianaeensis*. The red circles indicate (from left to right) MMR, PAH and KAP population units.

RAPDs analysis: Ten (10) ten-mer primers (University of British Columbia) were used in polymerase chain reactions (PCR) (5′-3′): 116: TAC GAT GAC G; 131: GAA ACA GCG T; 153: GAG TCA CGA G; 212: GCT GCG TGA C; 218: CTC AGC CCA G; 234: TCC ACG GAC G; 244: CAG CCA ACC G; 375: CCG GAC ACG A; 389: CGC CCG CAG T; 478: CGA GCT GGT C. PCR was performed in a volume of 15 µL containing 2 m*M* $MgCl_2$, 0.24 µ*M* of each dNTP, 15 ng BSA, 0.36 µ*M* primer, 0.3 ng genomic DNA, and 0.6 unit of *Taq* DNA polymerase (Sigma D4545). DNA amplification was performed using a thermal cycle of 94˚C (1.5 min), 38˚C (2 min), 72˚C (2 min) for initial strand separation, then 38 cycles of 91˚C (1 min), 38˚C (2 min), 72˚C (2 min), and a final cycle of 91˚C (1 min), 38˚C (2 min) and 72˚C (5 min) for final extension. Amplification products were analyzed by electrophoresis (72-75 V, 60 min) in 1.5% agarose gels in 1x TBE (tris-borate-EDTA) and detected by staining with ethidium bromide. Negative control reactions were run without DNA for all PCR amplifications to ensure reaction components were uncontaminated. Gels were digitized using Bio-Rad gel imaging system. Only markers that were unambiguous, well amplified, and reproducible in replicate tests were scored. Four specimens were not included in the final analyses due to inconsistency in amplification, namely ALI-A2, EKA-A10, LIA-A2 and PAH-A1.

Bands were scored as present (1) or absent (0), with co-migrating bands considered to be homologous loci. Absence of a band within individuals but present in others was assumed to indicate that this individual was a null homozygote; a present band indicated either a present homozygous locus or a heterozygote. Absence of a band from a population was assumed to indicate null homozygosity. The 148 polymorphic loci (71% of markers) were analyzed using principal co-ordinates analysis using the software, MultiVariate Statistical Package (MVSP3.1; Kovach Computing Services) using Gower similarity co-efficient (Gower 1966). UPGMA cluster analysis was also performed using both the Gower and the Nei & Li (1979) similarity coefficients. Percent polymorphic loci (%P) for all individuals combined and for each population (where more than one individual was analyzed) were determined as an estimate of the amount of genetic variation within the populations. Average expected heterozygosity was estimated for each locus in each population unit (*Hs*) and for all of the analyzed individuals combined (*Ht*) according to the equation:

$$H = 1 - (p^2 + q^2),$$

where *q* is the frequency of the null allele (absent band) and *p* is the frequency of the dominant (present) allele. The value obtained ranges from 0 to 1, with 0 inferring that all individuals are genetically identical (Harbin 2003). *Fst*, a measure of the amount of genetic differentiation occurring among populations, was calculated using the equation:

$$Fst = \frac{(Ht - Hs)}{Ht},$$

where an *Fst* value of 0 infers a high degree of gene flow among population, and a value of 1 indicates genetically isolated populations.

AFLP analysis: Amplified Fragment Length Polymorphism (AFLP; Vos et al. 1995) band profiles were generated following the protocol of the AFLP Analysis System I (Invitrogen Corp.). Restriction enzymes *EcoR* I and *Mse* I were used to digest 250 ng genomic DNA, and the ligation of adapters and preamplification reactions were performed following the published protocol. Selective amplification was undertaken using with nine primer pairs, with the *Eco* primers fluorescently labeled with WellRED dyes D3-PA or D4-PA (Proligo LLC) (Table 2).

The fluorescently labeled amplified fragments were accurately sized using the Beckman-Coulter CEQ8000 genetic analysis system with the inclusion of a size standard (DNA Size Standard Kit 400, Beckman-Coulter, Inc.) for calibration. Fragments differing by <1bp were scored as present or absent using the CEQ8000 fragment analysis software, and edited for accuracy. However, there was not sufficient variation found in the data to accurately assess the parentage of the MMR-C1 individual, and so this method will not be discussed further.

Table 2: Primers combinations tested on *Delissea waianaeensis* individuals.

	Mse-CAA	*Mse*-CAT	*Mse*-CAC	*Mse*-CAG	*Mse*-CTC
Eco-AAC	x				
Eco-AAG	x		x		x
Eco-ACC		x	x	x	x
Eco-AGC	x				

Results

Principal co-ordinates analysis (PCO) of the RAPDs data locus presence/absence data resulted in four distinct groupings that represented the population units and their geographic distribution (Figure 3). MMR-C1 was closely allied with both the Pahole and Kapuna individuals. The first axis of the PCO analysis separated the northern and southern population units, and the second axis tended to separate the more centrally located population units from those at the opposite ends of the range of the species in the Waianae Mountains. Cluster (UPGMA) analysis using both Gower and Nei & Li's coefficients also grouped MMR-C1 with both the Pahole and Kapuna wild individuals (Figure 4). MMR-C1 had the greatest overall similarity (0.78) to KAP-C2.

The most variable population units, both in terms of percentage polymorphic loci (%P) and measures of heterozygosity (*H*) were Pahole and ʻĒkahanui (Table 3). Polymorphism ranged from 6.7% (ALI) to 19.5% (PAH), with polymorphism across all individuals analyzed being 77%. However, some caution must be used in this interpretation, due to the very low sample numbers per population. Expected mean heterozygosity for all analyzed individuals was 0.27. The Palikea (ALI) population unit had the lowest estimated heterozygosity value (*H*=0.067) and highest F-statistic (*Fst*=0.75), indicating that this population unit is particularly isolated both genetically and geographically. Heterozygosity values for the other population units ranged from 0.128 (KAP) to 0.195 (PAH), and *Fst* values ranged from 0.28 (PAH) to 0.53 (KAP).

Discussion & recommendations for management actions

Delissea waianeensis displays a high level of genetic polymorphism when compared to other native Hawaiian species for which RAPDs analyses have been undertaken (see Harbin 2003, Kwon 1999). The average expected level of heterozygosity for the species was also high (*Ht*=0.27) when compared to other native Hawaiian species. The RAPDs data clearly indicates that the designated population units of *Delissea waianaeensis* are genetically distinct. Although the number of founder individuals is small (U.S. Army Garrison 2006), the high degree of genetic diversity held among populations emphasizes the need to preserve each population unit. As the parentage of the MMR-C1 individual could not be conclusively determined, it is recommended that it continue to be considered as a founder individual, potentially derived from the seedbank from the historic population in the area until further analysis is undertaken. MMR-

C1 was genetically similar to KAP and PAH individuals, and particularly KAP-C1. Given the proximity of MMR-C1 to reintroductions of KAP-A and KAP-B individuals, adding KAP-B individuals to future genetic analyses may help to resolve the question of parentage.

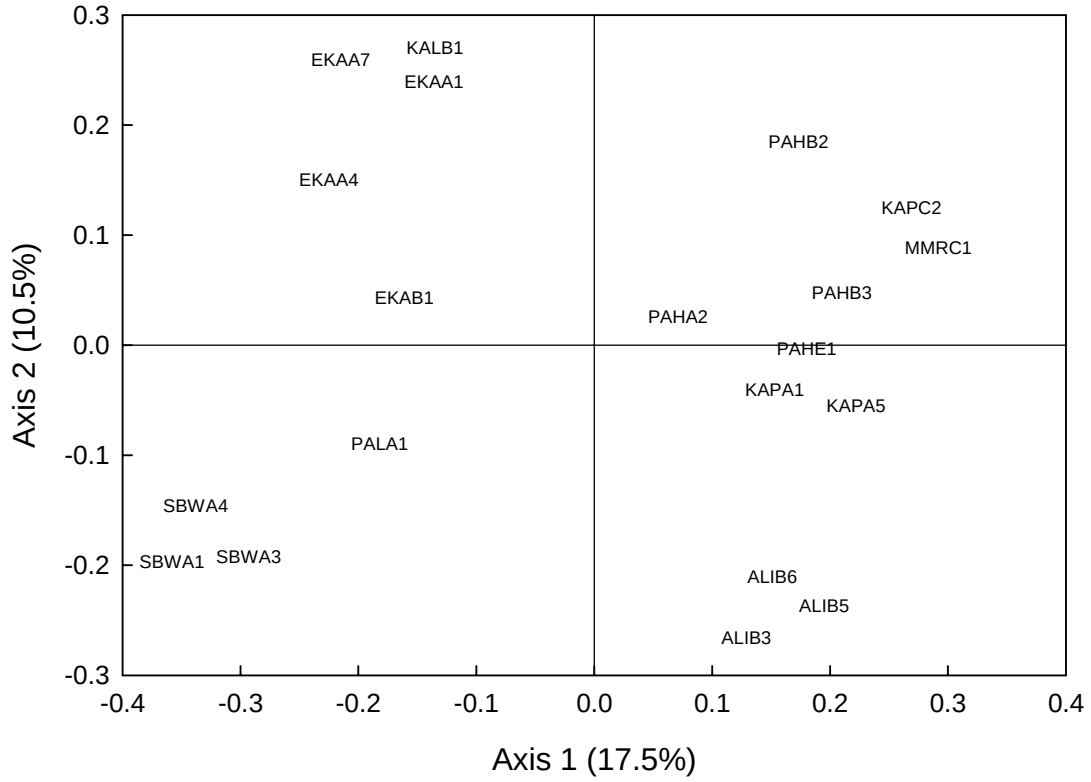

Figure 3. Principal co-ordinates analysis of the 148 RAPDs bands for 20 *Delissea waianaeensis* individuals using a Gower similarity coefficient matrix. Axes 1 and 2 represent 17.5% and 10.5%, respectively of the variation within the presence/absence data.

Table 3: RAPD statistics for each population unit of *Delissea wainaeensis*.

Population unit	Number individuals	%P	*H*	*Fst*
ALI	2	14	0.067	0.75
KAP	3	29	0.128	0.53
PAH	4	47	0.195	0.28
EKA	4	46	0.191	0.29
SBW	3	28	0.142	0.54
All	20	77	0.27	

Two other *Delissea* species known from the Waianae Mountains, namely *D. takeuchii* and *D. sinuata* are both presumed extinct (U.S. Army Garrison 2006), but hybridization between *D. takeuchii* and *D. waianaeensis* has been hypothesized (Lammers 2005). Including genetic material from collections of these species may help to clarify taxonomic issues associated with

the genetic differences observed in the RAPDs data for the geographically separated population units. Preserved herbarium specimens, however, can not be used for RAPDs analysis due to the degraded nature of the genetic material in the dried tissues. The development of microsatellite markers specific to this species may be needed to fully resolve the genetic variation within *Delissea waianaeensis.*

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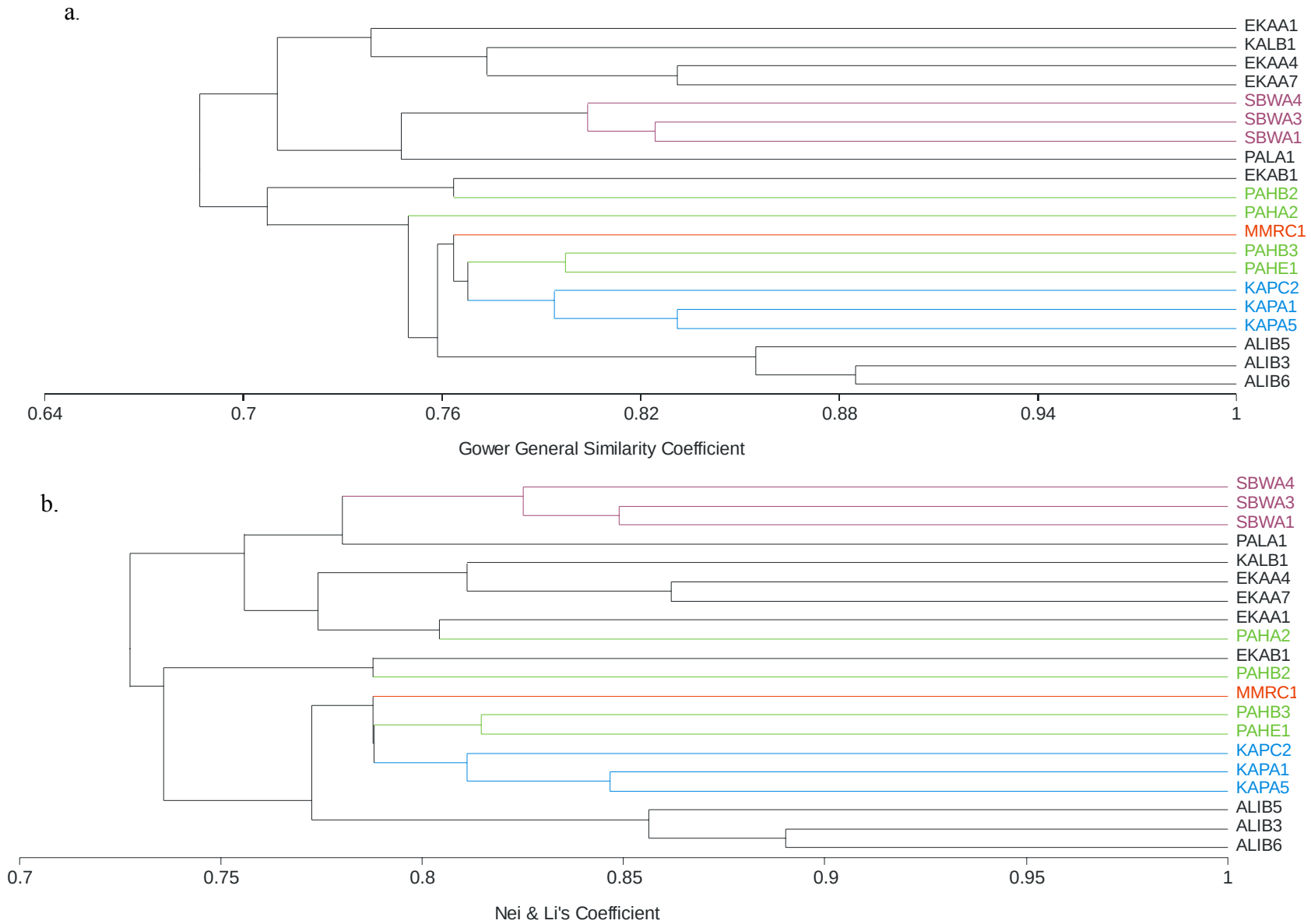

Figure 4. UPGMA (cluster) analysis of 148 RAPDs bands using (a) Gower and (b) Nei & Li's similarity coefficients. Differences in the two dendrograms can be accounted for by the fact that the Nei & Li coefficient measures only the shared presence of bands whereas the Gower coefficient also measures the shared absence of bands for two individuals.

Identification of invasive plant species on U.S. Army lands, 2008-2009.

Introduction

The goal of the U.S. Army's ecosystem management program is to conserve, protect and enhance the natural and cultural resources of Hawaii and comply with all applicable Federal and state laws and regulations while improving the Army's ability to conduct and maintain military readiness. In order to obtain this goal, a better understanding of the resources must be achieved to ensure proper management measures and decisions are made. Introduced plant taxa threaten endangered species and native ecosystems by altering habitat and disrupting community structure. Weedy plant species outcompete native plants for light, space and nutrients. As such, rapid identification of newly located invasive plant species is critical for their timely eradication on Army lands. The goal of this project was to accurately identify newly discovered invasive plant species found on U.S. Army lands using the resources of the Bishop Museum's *Herbarium Pacificum* (BISH).

Methods

During the period of 1 March 2008 to 31 May 2009, 80 plants specimens of unknown identity were collected from U.S. Army lands and deposited at the *Herbarium Pacificum*. Specimens that were fertile, new records, important distributional additions, or specimens expressing an important range of the plants morphological variability were mounted and accessioned into the BISH collections. If needed, images of the specimens were submitted to taxonomic experts for identification.

Results

Of the 80 plant specimens submitted to the Bishop Museum for identification, 59 specimens were identified to the species level or lower, 10 specimens could only be compared to known species, and 11 specimens were not able to be identified, largely due to the sterile nature of the material (Table 4). A further 6 specimens submitted prior to this funding were also confirmed during the current funding period. Total staff time dedicated to species identification and processing of specimens was 225 hrs. The BISH database currently records 20 of the Army collections during this period as having been mounted and accessioned into the herbarium.

Only three species identified were native (*Euyra sandwicensis*, *Metrosideros macropus*, and *Schoenoplectus tabernaemontani*); the remaining collections were all species introduced to the Hawaiian Islands. Of the collections made during 2008-2009, 11 taxa have not been previously recorded as naturalized in the Hawaiian Islands (New State Records) and 9 taxa have not been recorded as naturalized on Oahu (New Island (Oahu) Records). Of these, four (4) taxa have been confirmed as naturalized and will be published in the 2009 Bishop Museum Occasional Papers as New State Records (2 taxa) and New Island (Oahu) Records (2 taxa). Seven (7) further taxa, if identifications are confirmed in the future, are either New State or Island Records. Incipient weeds included *Paspalum virgatum*, *Digitaria ciliaris*, and *Axonopus fissifolius* (Poaceae), *Delairea odorata* (Asteraceae), *Corynocarpus laevigatus* (Corynocarpaceae), *Hyptis suaveolens* (Lamiaceae), *Tibouchina herbacea* (Melastomataceae), *Olea europaea* ssp. *cuspidata* (Oleaceae), and *Angiopteris evecta* (Marattiaceae). Species in the genera *Asparagus* and *Melaleuca* are also considered as incipient weeds, and other introduced species in the

Cupressaceae and Poaceae were difficult to identify, were frequently collected in native ecosystems.

Management actions

The rapid identification of unknown and potentially invasive species found on U.S. Army lands is critical for decision and management actions to quickly eliminate those introduced plant taxa that could irreparably damage native ecosystems. The significant number of New State and Island records discovered in the native ecosystems on Army lands is cause for concern, and demonstrates the importance of field surveys for introduced, invasive species. Digital images of unidentified specimens captured at the time of collection documenting characteristics of habit, flowers and fruits may further assist with the identification process.

Table 4: Collections made by U.S. Army personnel and identified by Bishop Museum staff from March 2008 to June 2009. Taxa not able to be identified due largely to material being sterile are highlighted in yellow; taxa highlighted in green require confirmation.

US Army #	Collection date	Family	Species	Comments
10	1/19/05	Theaceae	*Eurya sandwicensis*	Confirmed C. Imada 6/2009
28	12/8/05	Poaceae	*Agrostis hyemalis*	Confirmed N. Snow 5/2008; New State Record
35b	11/14/06	Smilacaceae	*Smilax bona-nox* L., vel. aff.	Confirmed C. Imada 6/2009
47	4/17/07	Myrtaceae	*Metrosideros macropus*	Confirmed C. Imada 6/2009
54	5/3/07	Poaceae	*Agrostis hyemalis*	Confirmed N. Snow 11/2008; New State Record
75	1/3/08	Poaceae	*Paspalum virgatum*	Confirmed N. Snow 11/2008; New State Record
84	3/4/08	Sapotaceae	*Sideroxylon persimile*	
85	5/6/08	Fabaceae	*Melilotus indica*	
86	5/6/08	Poaceae	*Digitaria ciliaris*	
87	5/6/08	Poaceae	*Bromus catharticus*	
88	5/6/08	Onagraceae	*Oenonthera kunthiana*	New Island Record?
89	5/6/08	Boraginaceae	*Heliotropium procumbens*	
90	5/6/08	Asteraceae	cf. Asteraceae	Sterile material
91	5/6/08	Brassicaceae	*Lepidium africanum*	New Island Record?
92	5/14/08	Oleaceae	*Olea europaea* ssp. *cuspidata*	New Island Record?
93	5/14/08	Poaceae	*Festuca rubra*	New Island Record?
94	5/20/08	Poaceae	*Paspalum virgatum*	
95	5/20/08	Iridaceae	*Trimezia martinicensis*	
96	8/4/08	Poaceae	Poaceae	Sterile material
97	8/7/09	Melastomataceae	*Tibouchina herbacea*	
98	8/13/08	Melastomataceae	*Tibouchina herbacea*	
99	9/15/08	Marattiaceae	*Angiopteris evecta*	
100	9/18/08	Nephrolepidaceae	*Nephrolepis cordifolia*	
101	9/17/08	Anacardiaceae	Anacardiaceae, cf. *Toxicodendron*	Sterile material
102	12/24/08	Orchidaceae	*Habenaria rodeiensis*	New Island Record?
103	1/3/09	Poaceae	*Paspalum paniculatum*	
104	1/3/09	Blechnaceae	*Blechnum orientale*	New State Record?
105	1/7/09	Pteridaceae	*Adiantum* 'Edwinii' hybrid	
106	2/9/09	Fabaceae	*Albizia* cf. *saponaria*	New Island Record if confirmed
107	1/28/09	Cupressaceae	*Callitris rhomboidea*	New State Record?
108	1/28/09	Cupressaceae	*Juniperus* cf. *bermudiana*	New Island Record if confirmed
109	1/28/09	Cupressaceae	*Callitris rhomboidea*	
110	2/18/09	Fabaceae	*Albizia lebbeck*	
111	2/18/09	Chenopodiacae	*Chenopodium carinatum*	
112	2/19/09	Poaceae	*Axonopus fissifolius*	
113	2/19/09	Poaceae	Poaceae	
114	2/19/09	Poaceae	*Digitaria fuscescens*	New Island Record?
115	2/19/09	Cupressaceae	*Cupressus* cf *lusitanica*	New State Record if confirmed
116	2/24/09	Pinaceae	*Pinus* sp.	
117	2/24/09	Cupressaceae	*Cupressus* sp.	

US Army #	Collection Date	Family	Species	Comments
118	2/24/09	Cupressaceae	*Cupressus* c.f. *lusitanica*	New State Record if confirmed
119	3/5/09	Fabaceae	*Albizia lebbeck*	
120	2/25/09	Poaceae	*Lolium* sp.?	
121	3/9/09	Corynocarpaceae	*Corynocarpus laevigatus*	
122	3/10/09	Poaceae	*Vulpia* sp.	Sterile material
123	3/10/09	Poaceae	*Anthoxanthum odoratum*	New Island Record?
124	3/10/09	Brassicaceae	*Cardamine flexuosa*	
125	3/10/09	Asteraceae	*Galinsoga quadriradiata*	
126	3/10/09	Caryophyllaceae	*Sagina japonica*	
127	3/10/09	Caryophyllaceae	*Cerastium fontanum* ssp. *triviale*	
128	3/9/09	Fabaceae	*Macrotyloma axillare* var. *glabrum*	
129	3/16/09	Myrtaceae	*Melaleuca* cf. *ericifolia*	New State Record if confirmed
130	3/16/09	Myrtaceae	*Melaleuca* cf. *ericifolia*	New State Record if confirmed
131	3/16/09	Myrtaceae	*Melaleuca* cf. *styphelioides*	New State Record if confirmed
132	3/16/09	Acanthaceae	cf. *Ruellia*	Sterile material
133	3/16/09	Crassulaceae	*Sedum* sp.	Sterile material
134	3/16/09	Crassulaceae	*Crassula multicava*	New Island Record?
135	3/16/09	Chenopodiaceae	*Chenopodium murale*	
136	3/16/09	Rubiaceae	*Richardia scabra*	
137	3/16/09	Lamiaceae	*Hyptis suaveolens*	
138	3/18/09	Hypnaceae	*Hypnum plumaeforme*	New State Record?
139	3/31/09	Scrophulariaceae	*Veronica serpyllifolia*	New Island Record?
140	4/8/09	Onagraceae	*Oenothera kunthiana*	
141	4/7/09	Malvaceae	*Sida acuta* ssp. *carpinifolia*	
142	4/7/09	Asteraceae	*Synedrella nodiflora*	New State Record?
143	4/23/09	Poaceae	*Schoenoplectus tabernaemontani*	
144	3/20/09	Blechnaceae	*Blechnum orientale*	New State Record?
145	3/20/09	Fabaceae	*Vigna luteola*	
146	3/20/09	Asteraceae	Asteraceae (*Sonchus*?)	
147	4/28/09	Poaceae	*Bromus hordeaceus*	
148	4/27/09	Scrophulariaceae	Scrophulariaceae	Submitted to specialist
149	5/13/09	Orchidaceeae	Orchidaceae	
150	5/13/09	Araliaceae	*Schefflera* cf. *elegantissima*	New State Record if confirmed
151	5/13/09	Liliaceae	*Asparagus* cf. *macowanii*	Need fertile material; New State Record if confirmed
152	5/13/09	Liliaceae	*Dianella caerula* var. *assera*	New State Record?
153	5/13/09	Corynocarpaceae	*Corynocarpus laevigatus*	
154	5/13/09	Asteraceae	*Delairea odorata*	New State Record?
155	5/13/09	Malvaceae	*Hibiscus ovalifolius*	New State Record?
156	5/13/09	Poaceae	*Lolium multiflorum*	
157	5/26/09	Fabaceae	*Albizia saponaria*	Confirmed New Island Record
158	5/26/09	Cupressaceae	*Callitris columellaris*	Confirmed New Island Record
159	5/26/09	Celastraceae	*Elaeodendron orientale*	New State Record?
160	5/26/09	Apiaceae	*Daucus pusillus*	
161	5/26/09	Poaceae	*Bromus hordeaceus*	

US Army #	Collection Date	Family	Species	Comments
162	5/27/09	Verbenaceae	*Verbena bonariensis*	
163	5/29/09	Nephrolepidaceae	*Nephrolepis* x*medlerae*	
164	5/30/09	Pteridaceae	*Pteris vittata*	